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Deregulation of c-*myc* Gene Expression in Human Colon Carcinoma Is Not Accompanied by Amplification or Rearrangement of the Gene

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The structure and expression of the c-*myc* oncogene were examined in 29 primary human colon adenocarcinomas. Dot blot hybridization of total RNA showed that 21 tumors (72%) had considerably elevated expression of c-*myc* (5- to 40-fold) relative to normal colonic mucosa. These data were corroborated by Northern blots of polyadenylated RNA, which showed a 2.3-kilobase transcript. Southern analysis of the c-*myc* locus in these tumors indicated the absence of amplification or DNA rearrangement in a 35-kilobase region encompassing the gene. In a parallel study, elevated expression of c-*myc* without amplification or DNA rearrangement was also observed in three of six colon carcinoma cell lines examined; in addition, unlike a normal colon cell line control, these three cell lines exhibited constitutive, high-level expression of the gene during their growth in cultures. These results indicate that elevated expression of the c-*myc* oncogene occurs frequently in primary human colon carcinomas and that the mechanism involved in the regulation of c-*myc* expression is altered in tumor-derived cell lines.

The c-*myc* gene is one of a small group of evolutionarily conserved cellular sequences known as oncogenes or proto-oncogenes, whose inappropriate expression or functional modification by mutation may be involved in carcinogenesis and tumor development (for reviews, see references 5, 6, 11, 52, and 53). Under normal circumstances, these genes are believed to play an important role in early development (36, 37) and may also be involved in the control of cell growth (30, 41), patterns of differentiation (22, 32, 42), and tissue repair processes (23, 34). The *myc* gene itself was first recognized as the principal oncogenic sequence of MC29, an acute retrovirus which causes sarcomas and carcinomas, as well as myelocytomatosis and B-cell lymphomas, in chickens (17, 24).

Significant evidence has been acquired in recent years to suggest that enhanced expression of c-*myc* leads to tumorigenesis in both avian and mammalian systems. Promoter insertion by integration of avian leukosis virus sequences adjacent to the c-*myc* gene in chickens (28, 38, 40) results in bursal lymphomatosis, a B-cell malignancy initiated by transcriptional activation of the host oncogene. Likewise, the reciprocal translocation of c-*myc* and immunoglobulin gene sequences characteristic of both murine plasmacytomas (1, 12, 35, 44, 48) and human Burkitt's lymphomas (1, 4, 14, 18, 26, 35, 48) frequently results in inappropriate expression of the translocated c-*myc* allele. The c-*myc* gene has also been reported to be amplified and significantly elevated in expression in the human promyelocytic leukemia cell line HL60 (10, 15) and the human colon cancer cell line COLO 320 (2). More recent reports of amplification in 1 of 5 human breast carcinoma cell lines (31) and 8 of 18 cell lines derived from human small-cell lung cancers (33) also suggest that an increase in gene copy number may occasionally serve to modify the expression of c-*myc* during tumorigenesis.

Nevertheless, high levels of expression of the c-*myc* gene which occur without concomitant amplification or rearrangement of the locus also appear to be an important consideration in oncogenesis. We recently completed a survey of c-*myc* structure and expression in 106 fresh hematopoietic malignancies in which we reported that 20 of those primary tumors (19%) had elevated expression of c-*myc* without apparent rearrangement or amplification of the gene (43). In this study of c-*myc* structure and expression in unselected primary human colon tumors, we now report that 72% of the 29 adenocarcinomas examined exhibited high levels of expression of the gene, again with no evidence of amplification or obvious genetic rearrangement in the vicinity of the locus. These data and the constitutive, high-level expression of the c-*myc* gene observed in several cell lines derived from colon carcinomas suggest that elevated expression of the gene occurs frequently in colon carcinomas in humans. The observed elevated levels of expression either may be the result of subtle genetic lesions at the c-*myc* locus itself or may represent a change in the mechanisms involved in the normal regulation of its expression. Our results are discussed in view of recent evidence of the involvement of the c-*myc* gene in cellular proliferation.

MATERIALS AND METHODS

Isolation of RNA and DNA from tumor samples and cultured cells. Colon tumor tissue and normal mucosa (near the margins of the resection) were removed by the attending pathologist immediately after surgery. The tumor sample and corresponding normal control were immediately frozen in liquid nitrogen and stored at −70°C until analyzed. At the time of analysis, ca. 1 cm^3 of tissue was minced and homogenized in guanidinium thiocyanate (Fluka) as described by Chirgwin et al. (9). DNA and RNA were recovered directly from tissue homogenates by centrifugation over a cushion of 5.7 M CsCl–0.1 M EDTA (pH 7.2)–6 μM aurintricarboxylic acid as described in detail previously (43). Cultured cells were lysed directly in guanidinium thiocyanate after removal of the medium and one or two washes with HDF (0.1% [wt/vol] glucose, 0.8% NaCl, 0.04% KCl, 0.02% EDTA; Puck's saline A with 0.02% EDTA). DNA and RNA were recovered as described above.

RNA and DNA blotting methods. Dot blotting of total

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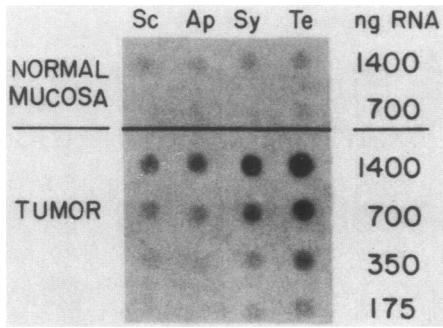

FIG. 1. Dot blot analysis of c-*myc* RNA levels in colon tumors relative to normal colonic mucosa. Total RNAs recovered from primary colon carcinoma tissue and normal colonic mucosa tissue from the same patient were bound to nitrocellulose and hybridized to ^{32}P-labeled pE3 probe (see Fig. 4, lower panel) by the method of Thomas (50). Samples Sc and Ap are representative of tumors with a moderately elevated expression of c-*myc* (ca. 5-fold increase), and samples Sy and Te are characteristic of tumors with a markedly elevated expression of c-*myc* (10-fold or more increase). Of 29 samples examined, 21 (72%) had increased levels of c-*myc* RNA 5-fold or more above normal.

RNAs and Northern blotting of polyadenylated RNAs were carried out as described previously (43), with the exception that 5% dextran sulfate was added to the hybridization mix. Restriction digests (New England BioLabs, Inc.) and electrophoresis of sample DNAs were also carried out as described previously (43). However, DNA transfers and Southern blot hybridizations (46) were carried out as follows. Before being transferred to nitrocellulose (Schleicher & Schuell Inc.), gels were soaked twice at room temperature in 0.5 N NaOH–1.5 M NaCl for 30 min, and then twice for 30 min in 0.5 M Tris-hydrochloride (pH 7.0)–1.5 M NaCl. Transfer to nitrocellulose was done for 12 to 18 h in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Blots were washed in 6× SSC, baked for 2 h at 80°C in vacuo, and then prehybridized for 30 min at room temperature in 10× Denhardt solution (16) containing 0.4% sodium dodecyl sulfate. Prehybridized blots were baked for 30 min at 80°C in vacuo and then hybridized overnight at 37°C in a solution containing 50% formamide, 5× SSC, 5× Denhardt solution, 0.1% sodium dodecyl sulfate, 0.1% sodium PP_i, 50 mM NaH_2PO_4 (pH 7.0), and denatured salmon sperm DNA (100 μg/ml) containing 3×10^6 to 4×10^6 cpm of ^{32}P-labeled, heat-denatured DNA probe per ml. Hybridized blots were washed three times for 15 min at room temperature in 2× SSC–0.1% sodium dodecyl sulfate–0.1% sodium PP_i and then two times for 60 min at 65°C in 0.2× SSC–0.1% sodium dodecyl sulfate–0.1% sodium PP_i. Blots were exposed overnight at −70°C in the presence of an intensifying screen (Cronex Lightning-Plus; E. I. du Pont de Nemours & Co., Inc.).

DNA probes. DNA probes for RNA and DNA blotting were derived from a 13-kilobase (kb) *Eco*RI clone (λhR1-15) of the human c-*myc* gene isolated as described previously (43). In general, a 1.4-kb *Cla*I-*Eco*RI subclone (pE3) encompassing the third exon of c-*myc* was used for hybridization of dot blots and both Northern and Southern blots. A second probe (ph7) derived from the extreme 5′ end of λhR1-15 (more than 7 kb upstream from exon 1) was also used to search for rearrangements in sequences adjacent to the c-*myc* locus (see Fig. 4).

Cell cultures. Normal colon and colon carcinoma cell lines were obtained from the American Type Culture Collection. Cells were routinely grown in RPMI medium supplemented with 13% fetal bovine serum and 1.0 mM sodium pyruvate. For the purpose of splitting and propagating cultures, cells were washed twice in HDF (Puck's saline A with 0.02% EDTA; see above) and then treated with HDF containing 0.04% trypsin for 10 min at 37°C. The colon carcinoma cell lines used were frequently refractory to trypsin treatment, in which case the cells were also scraped after a 10-min incubation with HDF containing 0.04% trypsin.

The colon cell lines used in these studies were as follows, with the repository designation preceding the corresponding cell line name. The normal colon (fibroblast) cell lines were CRL 1459, CCD-18Co; CRL 1539, CCD-33Co; and CRL 1541, CCD-112CoN. The colon carcinoma cell lines were CCL 221, DLD-1; CCL 222, COLO 205; CCL 224, COLO 201; CCL 233, SW1116; CCL 237, SW 948; and CCL 238, SW 1417.

RESULTS

Frequent elevated expression of c-*myc* in human colon carcinomas. Total RNAs recovered from colon carcinoma samples and adjacent normal colonic mucosa were initially screened for expression of the c-*myc* gene with dot blots (50). In each case, 1.4 to 1.5 μg of total RNA and twofold serial dilutions of that amount were bound to nitrocellulose and hybridized to ^{32}P-labeled pE3 probe (a 1.4-kb *Cla*I-*Eco*RI fragment of the human c-*myc* gene encompassing exon 3). The expression of c-*myc* in tumor tissue was judged relative to that in corresponding normal mucosa from the same patient (Fig. 1). Although 8 of the 29 tumors examined (28%) had essentially no increase in c-*myc* RNA levels (less than 2-fold), 10 (34%) had moderately elevated expression (ca. 5-fold times normal, e.g., samples Sc and Ap), and another 11 (38%) had a marked increase in expression (ca. 10-fold or more times normal, e.g., samples Sy and Te). Of those 11 tumors, 2 had 20- and 40-fold greater amounts of message than normal mucosa. Consequently, 72% of the tumors examined displayed an obvious increase in c-*myc* RNA levels.

Shown in Fig. 2 are typical examples of tumor samples exhibiting no increase in c-*myc* expression (samples Tr, Ba, and We), moderately elevated expression (samples Sp and

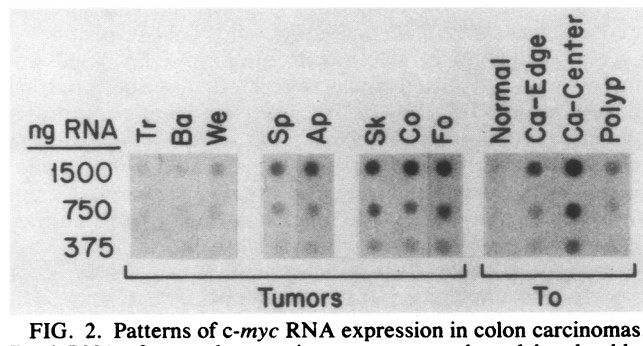

FIG. 2. Patterns of c-*myc* RNA expression in colon carcinomas. Total RNAs from colon carcinomas were evaluated by dot blot hybridization as described in the legend to Fig. 1. Samples Tr, Ba, and We are representative of 8 of 29 tumors with no apparent increase in c-*myc* RNA levels. Samples Sp and Ap represent 10 of 29 tumors (34%) with moderately increased expression, and samples Sk, Co, and Fo represent 11 of 29 tumors (38%) with significantly increased expression. Sample To shows the differential expression of the gene in central and peripheral portions of the tumor (Ca, carcinoma), as well as in a nearby polyp.

Ap), or markedly elevated expression (samples Sk, Co, and Fo). Of particular interest in Fig. 2 is an additional set of samples derived from a single patient, To. An analysis of tissue from adjacent normal colonic mucosa, both the peripheral and central portions of the tumor, and a polyp recovered from an area near the tumor was carried out. Tissue from the central portion of the tumor exhibited a marked elevation of c-*myc* expression, whereas peripheral tumor tissue exhibited a somewhat lower, although demonstrably elevated, level of c-*myc* RNA. The nearby polyp had a moderately elevated level of c-*myc* RNA which was lower than that found in peripheral tumor tissue but clearly higher than that detected in normal mucosa.

Confirmation of levels of expression and determination of transcript size were accomplished with Northern blots of polyadenylated RNAs hybridized to ^{32}P-labeled pE3. Densitometric evaluation of Northern blots (data not shown) confirmed estimates of expression levels previously derived from the dot blot analysis. This indicates that the increase in c-*myc* RNA levels frequently observed was not a consequence of a more general increase in mRNA synthesis. The upper panel of Northern blots in Fig. 3 corresponds to several of the tumor tissue-normal tissue pairs shown in Fig. 1 and 2. A band corresponding to a c-*myc* transcript of ca. 2.3 kb was observed for both tumor tissue and normal mucosa tissue in samples with nonelevated (sample Tr), moderately elevated (samples Sc and Ap), and markedly elevated (sample Sy) c-*myc* levels. Although the size of this transcript appeared to be the same in both tumor tissue and normal mucosa tissue, we have not investigated any possible change in the differential use of promoters such as has been reported for some Burkitt's lymphomas (49).

To control for the quantity and quality of polyadenylated RNAs used in these experiments, we rehybridized Northern blots to a ^{32}P-labeled human β_2-microglobulin probe (47). As shown in the lower panel of Fig. 3, comparable signals for the β_2-microglobulin transcript were observed for all tumor tissue-normal tissue pairs, indicating that the less intense signals for c-*myc* observed in normal controls were not due to inefficient selection, poor transfer, or RNA degradation. These data corroborate evidence of increased levels of c-*myc* RNA in the tumor samples examined.

Lack of amplification or rearrangement of the c-*myc* locus in tumors with elevated expression of the gene. DNA recovered from tumor tissue was analyzed for obvious rearrangement or amplification of the c-*myc* locus. DNAs were digested with a variety of restriction enzymes, transferred to nitrocellulose, and hybridized to one of the two different ^{32}P-labeled probes shown in Fig. 4 (lower panel). Digestion of DNAs with *Kpn*I (panel a), *Eco*RI (panel c), and *Hin*dIII-*Eco*RI (panel d), followed by hybridization of the resulting Southern blots with ^{32}P-labeled probe pE3, provided no evidence of gene rearrangement of the immediate c-*myc* locus or DNA sequences as much as 7 kb upstream and 10 to 11 kb downstream from the coding region. Digestion with *Kpn*I (panel b) and subsequent hybridization of Southern blots with ^{32}P-labeled probe ph7 (a 1.2-kb *Eco*RI-*Bgl*II fragment at the extreme 5′ end of the λhR1-15 clone of c-*myc*) provided similar evidence of the structural integrity of 5′-flanking sequences as much as 18 to 19 kb upstream from the coding region of c-*myc*. Additional evaluations done with *Hin*dIII, *Sac*I, *Xba*I, *Xho*I, and a number of double digests designed to examine smaller regions of the c-*myc* locus more closely also failed to reveal any obvious genetic changes (data not shown). Normal mononuclear leukocyte DNA (OKTA) was used as a control. Consequently, no rearrangement of DNA sequences within a ca. 35-kb region encompassing the coding sequence of c-*myc* was observed in any of the tumor samples, including those with significantly increased levels of c-*myc* RNA. Whether or not point mutations or small deletions or insertions which affect c-*myc* expression have occurred cannot be determined by this analysis.

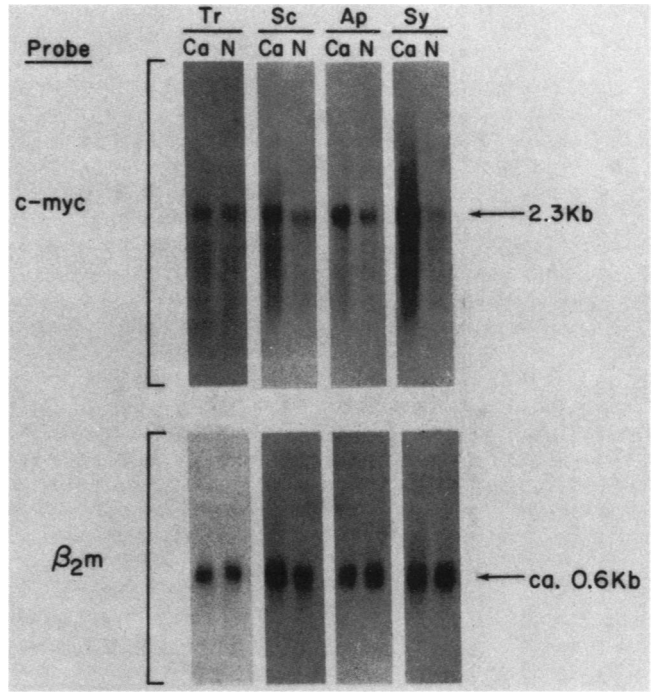

FIG. 3. Northern blots of c-*myc* RNA from colon carcinoma samples and normal controls. Samples (5 μg) of polyadenylated RNAs from tumor tissue and corresponding normal mucosa tissue were fractionated on 0.8% agarose gels, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled human c-*myc* probe (pE3, upper panel) to determine c-*myc* transcript size. Northern blots were rehybridized to a ^{32}P-labeled β_2-microglobulin probe (β_2m, lower panel) (47) to assess the quality of the RNA used. Ca and N, Carcinoma tissue and normal tissue, respectively.

To facilitate the detection of any amplification of sequences in the region examined in these DNA studies, reasonable care was exercised in quantitating DNA concentrations and in certifying the efficiency of DNA transfer to nitrocellulose. The individual lanes in each Southern blot shown in Fig. 4 represent equivalent amounts of DNA from each sample (5 μg). It is evident that none of the tumors shown or any of the colon DNAs examined to date exhibited significant amplification of sequences in or around the c-*myc* locus. These results eliminate gene amplification as a cause for the elevated expression of c-*myc* RNA observed in two-thirds of these primary tumors.

Constitutive, high-level expression of c-*myc* in many colon carcinoma cell lines. Recent work in several laboratories (3, 7, 25, 30) has demonstrated an apparent responsiveness of c-*myc* RNA levels to mitogenic stimulation, suggesting that cell proliferation, or more specifically entry into the cell cycle from G_0, may result in a transient increase in the transcription of this gene. We examined c-*myc* mRNA levels in a series of cell lines derived from both normal colon and colon carcinomas, and in two cases (one normal line and one carcinoma line), we looked specifically at the levels of message in growing cell populations.

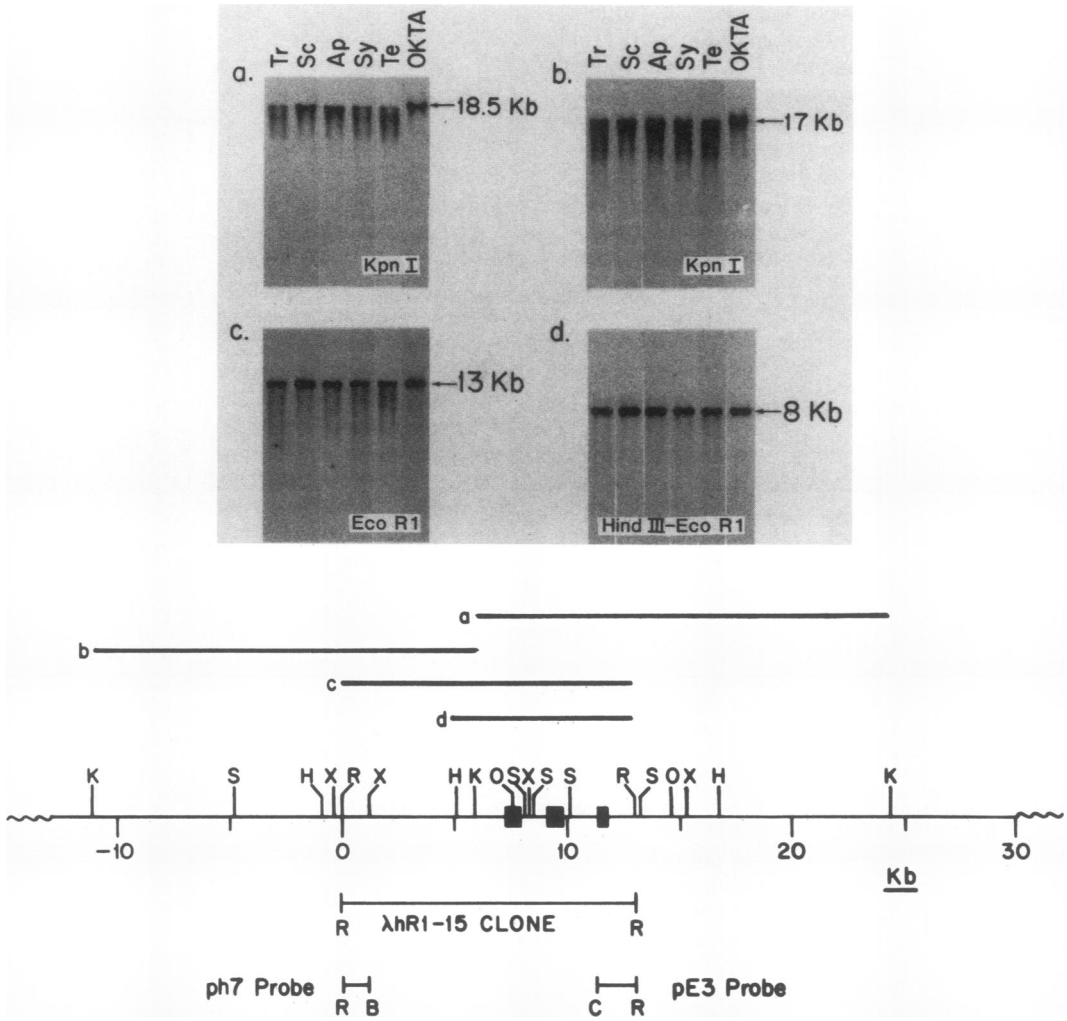

FIG. 4. Southern blot analysis of the c-*myc* locus in DNAs from colon carcinoma samples and a normal control. Samples (5 μg) of tumor DNAs and OKTA (see text) were digested, fractionated on 1% agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled probes by the method of Southern (46) with the modifications described in the text. The tumors had c-*myc* RNA levels ranging from not elevated (Tr) to moderately (Sc and Ap) or markedly elevated (Sy and Te). Digests in panels a, c, and d were hybridized to ^{32}P-labeled pE3 probe. They reflect an analysis of the fragments shown above the map of the human c-*myc* locus around the coding region and on the 3′ side of the gene. Digests in panel b were hybridized to ^{32}P-labeled ph7 probe, which detects the 5′ sequences adjacent to c-*myc*. Subclones used as probes were derived from the λhR1-15 clone of human c-*myc* described previously (43). Restriction sites within the λhR1-15 clone on the genomic map are complete for the restriction enzymes indicated; however, sites mapped in the 5′- and 3′-flanking regions are incomplete. Abbreviations for the restriction enzymes are as follows: B, *Bgl*II; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; O, *Xho*I; R, *Eco*RI; S, *Sac*I; and X, *Xba*I. Exons 1, 2, and 3 of c-*myc* are indicated as black boxes. Their approximate positions are based on the published sequence of the gene (4, 21).

Initially, as shown in the upper panel of dot blots in Fig. 5, c-*myc* RNA levels in confluent cells were determined. Whereas the total RNAs of cell lines derived from normal colonic mucosa (CRL 1539, CRL 1541, and CRL 1459) exhibited very low levels of the c-*myc* transcript, several cell lines derived from colon carcinomas (CCL 233, CCL 237, and CCL 238) had a markedly increased expression of the gene (more than a 10-fold elevation). Still other carcinoma cell lines (COLO 205, COLO 201, and CCL 221) had low levels of c-*myc* RNA, comparable to those measured in the normal controls. Consequently, the high levels of c-*myc* mRNA observed in half of these carcinoma cell populations seemed to parallel our finding of elevated expression in a large number of the primary tumor samples described above.

In the lower panel of Fig. 5, Northern blots of polyadenylated RNAs from these cell lines confirmed the levels of expression indicated by dot blot analysis and clearly demonstrated the synthesis of a 2.3-kb transcript. The Northern blots shown were rehybridized to a ^{32}P-labeled β_2-microglobulin probe (47) as described above as a control for RNA integrity. All of the samples analyzed contained roughly equal amounts of intact, hybridizable β_2-microglobulin mRNA. Southern blots of DNAs from all of these cell lines restricted with *Eco*RI showed no indication of either gene rearrangement or amplification (data not shown).

In addition to making static measurements of mRNA levels in these cells at confluence, we also looked at the relationship between growth in culture and c-*myc* expression in two cell lines as they developed from initially seeded cultures to confluent ones. Normal colon cell line CRL 1459, which expresses very low levels of c-*myc* at confluence, and colon carcinoma cell line CCL 233, which expresses signifi-

cantly elevated levels, were seeded from confluent cultures at ca. 10% densities and permitted to grow normally. Cells were lysed in guanidinium thiocyanate at various times throughout the growth of the cultures to isolate RNA.

Although normal colon cell line CRL 1459 contains low levels of c-*myc* RNA when quiescent (Fig. 5), it did indeed exhibit transient, enhanced expression of the gene just after splitting at a time when the cells were presumably reentering the cell cycle after a period of little growth. This enhancement in mRNA levels decreased relatively rapidly as the cells continued to grow (Fig. 6a). Within 70 h, which represents approximately one doubling in this experiment (Fig. 6c), the c-*myc* RNA content returned to the low level previously observed at confluence, although the cultures were just ca. 20% confluent at this time. These data indicate that an increase in c-*myc* expression does occur as proliferation of these cells is reinitiated but that the elevated levels of message are significantly reduced when a stable, growing culture has been established. This suggests that asynchronous growth per se is not responsible for significantly elevated levels of message and that enhanced expression is a result of the transition from quiescence to a growing state for these cells.

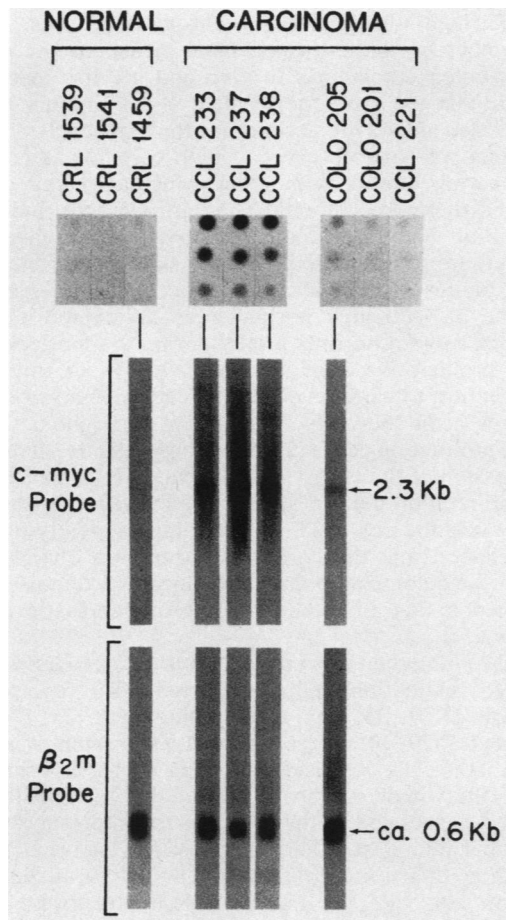

FIG. 5. Dot (upper panel) and Northern blot (lower panel) analyses of c-*myc* expression in colon cell lines. Total RNAs recovered from both normal (fibroblastic) colon cell lines (CRL 1539, CRL 1541, and CRL 1459) and colon carcinoma cell lines (all others shown) were screened for c-*myc* transcripts by dot blot analysis as described in the legend to Fig. 1. A 1.5-μg sample of total RNA and twofold serial dilutions of that amount were analyzed. Northern blot analysis of 5-μg samples of polyadenylated RNAs from selected cell lines was carried out as described in the legend to Fig. 3. β_2m, β_2-Microglobulin.

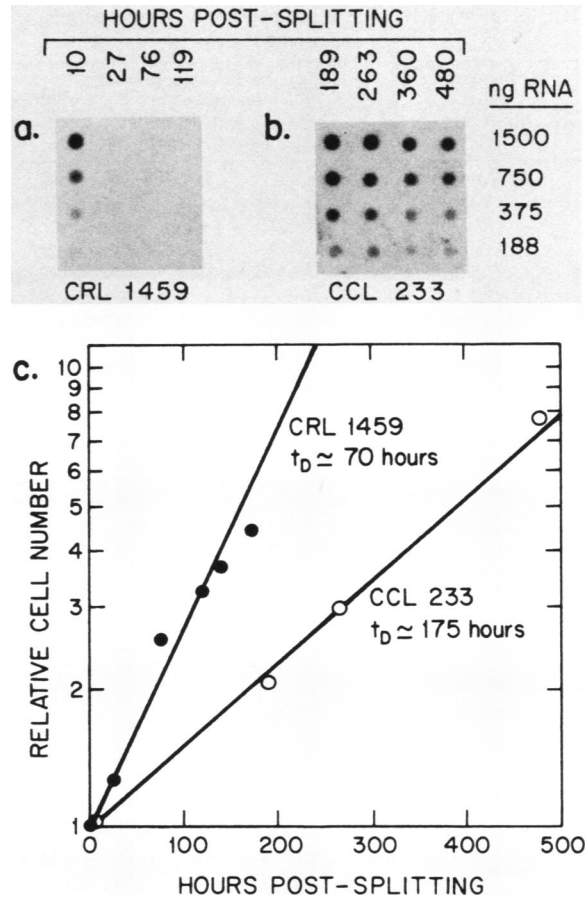

FIG. 6. Levels of c-*myc* RNA in normal colon (CRL 1459) and colon carcinoma (CCL 233) cell lines during growth in cultures. Confluent cultures of normal (fibroblastic) colon cell line CRL 1459 and colon carcinoma cell line CCL 233 were treated with trypsin, seeded at ca. 10% confluence, and grown as described in the text. RNA was recovered from cells lysed in guanidinium thiocyanate at various times. A 1.5-μg sample of total RNA and twofold serial dilutions of that amount were analyzed for c-*myc* transcripts by dot blot analysis. The time points chosen reflect three periods of growth after splitting: early growth, when cells have reattached to the dish and are 5 to 10% confluent (CRL 1459, 10 h; CCL 233, 189 h); midgrowth, when cells are 15 to 25% confluent (CRL 1459, 27 and 76 h; CCL 233, 263 and 360 h); and late growth, when established cultures are 40 to 70% confluent (CRL 1459, 119 h; CCL 233, 480 h). Panels a and b show the levels of c-*myc* RNA in CRL 1459 and CCL 233, respectively, measured at the indicated time points after splitting. Panel c is a growth curve for these cell lines done in conjunction with the expression study. t_D, Doubling time.

In contrast, the levels of c-*myc* RNA in colon carcinoma cell line CCL 233, which are elevated in confluent cultures (Fig. 5), remained consistently high throughout the proliferative phase after splitting. These elevated levels of message were not diminished significantly during the growth of these cells in cultures (Fig. 6b), the population doubling time being ca. 175 h (Fig. 6c). Consequently, steady-state mRNA levels in the slow-growing carcinoma cell population do not appear to be subject to the same constraints imposed on the more rapidly growing normal cell population. The reason for this

TABLE 1. c-*myc* RNA levels in unselected primary human colon carcinomas

No. of tumor samples	Increase in c-*myc* RNA level (fold)[a]	No. confirmed by northern blots	No. with rearranged or amplified c-*myc* locus[b]	% of tumors examined
8	None	2	0	28 (No increase in c-*myc*)
10	5	4	0	34 (Moderately elevated c-*myc*)
9	10	9	0	38 (Markedly elevated c-*myc*)
1	20	1	0	
1	40	1	0	

[a] Based on dot blots of total RNA hybridized to a c-*myc* probe. Increases were judged relative to levels in normal colonic mucosa (Fig. 1).
[b] Based on Southern blots analysis (Fig. 4).

change in regulation is not apparent, as neither amplification nor gross rearrangement of the c-*myc* locus occurred in this carcinoma cell line.

DISCUSSION

We have shown that a considerable number of primary human adenocarcinomas of the colon exhibit high levels of expression of the c-*myc* gene without having undergone apparent rearrangement or amplification of the locus (Table 1). Of 29 tumors examined, 21 (72%) had c-*myc* RNA levels at least 5-fold and as much as 40-fold higher than those in normal colonic mucosa but had no obvious genetic changes in the normal configuration of the gene. Consequently, genetic rearrangement of the locus as has been reported in the case of Burkitt's lymphoma (1, 4, 14, 18, 26, 35, 43, 48) does not appear to be frequent in or necessary for high levels of expression in neoplastic tissue. To date, we have examined more than 200 unselected primary human tumors (leukemias, lymphomas, and colon carcinomas), and with the exception of 2 of 11 lymphomas studied (43), we have found no evidence of obvious genetic rearrangement, even though more than 50 cases of significantly elevated levels of c-*myc* RNA were observed. Similarly, in none of more than 200 primary tumors examined was amplification associated with high levels of c-*myc* transcripts, suggesting that the amplification of this gene reported in the case of colon carcinoma cell line COLO 320 (2) and other cell lines derived from breast (31) and lung (33) tumors may be a late event in neoplastic progression or a consequence of establishment in cultures. The results of our examination of many primary human tumors suggest that neither rearrangement nor amplification of c-*myc* are necessary primary genetic changes linked to its enhanced expression.

We have also shown that the pattern of c-*myc* regulation during growth in cultures of a colon carcinoma cell line (CCL 233) is clearly different from that of a normal colon-derived fibroblast cell line (CRL 1459). The normal cells exhibit a dramatic increase in numbers of transcripts when growth is reinitiated by reseeding confluent cultures at a reduced density. The number of c-*myc* transcripts then returns to low, basal levels before the second cell division (doubling time, ≈70 h). Consequently, the induction of c-*myc* expression in these cells appears to accompany the release from G_0 and reentry into the cell cycle. A similar, transient increase in c-*myc* RNA levels has also been observed in normal fibroblasts stimulated by platelet-derived growth factor (30), in serum-stimulated 3T3 cells (7), and in mitogen-stimulated lymphocytes (29).

In contrast to this pattern of expression in CRL 1459, the expression of c-*myc* in a growing population of cells from colon carcinoma cell line CCL 233 is substantially different. Levels of c-*myc* transcripts at confluence are high and essentially equivalent to those in normal cells during the transition from G_0 to G_1; however, these elevated levels are not reduced significantly during subsequent growth, even after two or three population doublings (doubling time, ≈175 h). It is important to note that this persistent elevation of transcript levels sustained during growth in cultures is not correlated with an increased rate of proliferation and that constitutive expression of c-*myc* has also been observed in chemically transformed mouse cells (7). These results indicate that there is no direct correlation between the apparent rates of cell cycling (CRL 1459, 70 h; CCL 233, 175 h) and the dynamic levels of c-*myc* transcripts (CRL 1459, low; CCL 233, high) observed in asynchronous cultures.

A number of studies which have measured the growth rates of colon carcinomas in vivo and in vitro lead us to conclude that the proliferative state of the primary tumors per se is also unlikely to account for the high levels of c-*myc* transcripts we have observed. Colon carcinomas are very slow growing tumors with greatly increased cell cycling times. Furthermore, although they are variable, indices of proliferation and cell production rates which have been measured are not regarded as being significantly increased relative to those in normal colonic mucosa (for a review, see reference 8). Although normal cells do exhibit elevated levels of c-*myc* transcripts briefly during the first cell cycle after a proliferative stimulus, the numbers of transcripts quickly return to basal levels during subsequent growth and have now been shown to be invariant throughout the cell cycle in proliferating cells (27, 51). Consequently, high levels of expression of the c-*myc* gene appear to be associated not with proliferation per se (20) but rather with the transition from G_0 into the cell cycle. These findings greatly increase the likelihood that the enhanced expression of the c-*myc* locus in the colon carcinomas examined is a tumor-specific phenomenon that is not an artifact of neoplastic growth rates.

Studies citing the transient induction of c-*myc* expression linked to reinitiation of growth have been reported for fibroblasts (3, 7, 25, 30), regenerating liver (23, 34), and lymphocytes (29, 30), suggesting that a spectrum of normal, differentiated cells exhibit the behavior we have observed in CRL 1459, which is fibroblastic in origin. Constitutive, high-level expression of the gene has not been reported for any normal adult tissue. The tumor tissue we have analyzed, although epithelial in origin, is undoubtedly a mixed population of cells like the normal colonic mucosa controls examined. However, we have no indication at the present time that the high-level, tumor-specific expression of c-*myc* we have observed is a normal function of the cell type from which the neoplasms originated. Furthermore, our examination of histopathologic data on the primary tumors studied indicates no apparent histologic or morphologic difference between those colon carcinomas with high levels of c-*myc* transcripts and those without (P. G. Rothberg et al., Br. J. Cancer, in press).

It is not unreasonable to suspect that constitutive, elevated expression of the c-*myc* gene contributes to altered growth control in these tumor cells. It has been suggested that relaxation of the normal schedule of c-*myc* expression, rather than absolute levels of transcription, may be responsible for some aspects of the loss of growth control (7, 30, 43). Deregulated c-*myc* gene expression may contribute to transformation by preventing exit from the cell cycle into G_0 (30). We view the constitutive, apparently deregulated expression of c-*myc* in CCL 233 as being pertinent to the data we have accumulated in our analysis of primary colon tumors. Like CCL 233, more than two-thirds of the primary colon tumors examined exhibit elevated levels of c-*myc* transcripts which cannot be related to obvious structural changes at the genetic locus, suggesting that the regulation of c-*myc* transcription or RNA turnover has been affected in these tumors.

Therefore, we propose that the high levels of c-*myc* transcripts found in these colon tumors are the result of an acquired, tumor-specific change which disrupts the normal regulation of c-*myc* expression. Although our data indicate that amplification and gross rearrangement of the c-*myc* locus are not the cause of such an event, the possible presence of point mutations or small deletions and insertions at the locus which affect its expression cannot be excluded. It is also possible that the regulation of the gene has been disrupted through mutation of a second genetic locus such as has been proposed to regulate its transcription through a *trans*-acting factor (13, 19, 39, 45). We believe that these findings could indicate the existence of a subset of primary malignancies which may have undergone deregulation of c-*myc* expression during tumorigenesis owing to a genetic change at a second locus.

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